Are Pheromones Key to Unlocking Cryptic Lizard Diversity?

Stephen M. Zozaya,^{1,*} Megan Higgie,¹ Craig Moritz,² and Conrad J. Hoskin¹

1. College of Science and Engineering, James Cook University, Townsville, Queensland 4811, Australia; 2. Research School of Biology, Australian National University, Canberra, Australian Capital Territory 2601, Australia

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ABSTRACT: Animals use mating traits to compete for, attract, and choose mates. Because mating traits influence mate choice, the divergence of mating traits between populations can result in reproductive isolation. This can occur without associated morphological divergence, producing reproductively isolated cryptic species that are visually indistinguishable. Thus, identifying the mating traits in morphologically conservative groups is key to resolving diversity and speciation processes. Lizards contain many such groups, with phylogeographic studies often revealing highly divergent but morphologically cryptic lineages within species. Considering that cryptic lizard species can be sympatric but morphologically indistinguishable, we hypothesize that candidate species will exhibit divergent pheromones and that pheromones will have typically diverged more than morphology. To test this, we used gas chromatography to characterize pheromones (epidermal pore secretions) from 10 genetically divergent lineages of the Bynoe's gecko (*Heteronotia binoei*) species complex in northern Australia. Multivariate analyses of pheromone blends and morphology indicate that pheromones are lineage specific and have diverged relatively more than morphology. Such specificity suggests that pheromones influence behavioral isolation in this morphologically conservative lizard radiation. These results suggest that pheromone data may unlock the tremendous cryptic diversity currently being uncovered in many lizard groups.

Keywords: cryptic species, signaling trait, chemical communication, trait divergence, gecko, *Heteronotia binoei*.

Introduction

Phylogeographic studies reveal that many so-called species are actually complexes of morphologically similar but deeply divergent genetic lineages. With further investigation, some of these are found to be undescribed species, often referred to as cryptic species. Resolving cryptic species is important because they compose a significant and largely undescribed proportion of global biodiversity (Bickford et al. 2007). Cryptic

species often reflect species classification biased by the limits of human perception. We rarely find cryptic species in birds, a group that has diversified in color and vocalization, but instead find them more often in groups such as reptiles, crustaceans, and onychophorans (Pérez-Ponce de León and Poulin 2016). Many cryptic species represent genetically divergent allopatric populations; however, some cryptic lineages exhibit little or no gene flow when in sympatry or secondary contact (e.g., Stuart et al. 2006; Amato et al. 2007; Singhal et al. 2018; Pinto et al. 2019). Considering this, these species are unlikely to be cryptic to each other. More likely, the traits that facilitate behavioral isolation in these groups are difficult to observe. Indeed, the diversity of many groups has been better resolved by technologies that facilitate analysis of cryptic signaling traits, such as pheromones in ermine moths (Löfstedt et al. 1991), ultrasonic communication in lacewing insects (Wells and Henry 1998), and weak electrical signals in mormyrid fish (Hopkins and Bass 1981). Understanding cryptic species diversity therefore requires knowledge of the traits that the animals themselves use to discriminate between each other.

Animals use mating traits to compete for, attract, and choose mates, which involves both a mating signal and an associated preference for that signal (Endler 1989; Andersson 1994). The divergence of signals and preferences among populations can result in premating isolation, involving reduced attraction between members of different populations or species that prevents courtship or mating (Mayr 1963; Ptacek 2000; Hoskin et al. 2005; Ritchie 2007). When the respective signals are not visual, mating traits can diverge between populations without any associated morphological changes, resulting in genetically isolated but visually similar species that differ primarily in their mating signals (Panhuis et al. 2001). Considering this, we expect cryptic species to be more common in groups that do not typically exhibit visual or auditory signaling. This is not a new idea: Ernst Mayr postulated that cryptic species (referred to by Mayr as “sibling species”) would be more common in groups in which chemical cues are more highly developed than the sense of vision (Mayr 1963). We predict that this is true not just about chemical signals but also about any signaling trait not easily per-

* Corresponding author; email: stephen.zozaya@my.jcu.edu.au.

ORCID: Zozaya, <https://orcid.org/0000-0003-3451-3552>; Higgie, <https://orcid.org/0000-0002-2397-0240>; Moritz, <https://orcid.org/0000-0001-5313-7279>.

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ceived by humans. Conversely, one might expect groups already known to harbor substantial morphologically cryptic diversity to employ nonvisual (cryptic) signals as mating traits.

Squamate reptiles (lizards and snakes) are the most species-rich clade of terrestrial vertebrates, with more than 10,000 described species worldwide (Uetz and Hošek 2018). In many taxa there is strong sexual dimorphism for breeding colors or ornamentation, with these traits often differing between species (e.g., *Anolis*; Losos 2009). But many other taxa lack such obvious traits, and for these taxa genetic studies often reveal high levels of cryptic diversity. Lizards and snakes are a standout group among vertebrates in terms of the number of cryptic species and, when corrected for research effort, are ranked highly for cryptic diversity among all animal groups (Pérez-Ponce de León and Poulin 2016). Geckos appear to harbor especially high levels of cryptic diversity (Werneck et al. 2012; Domingos et al. 2014; Garcia-Porta et al. 2017; Moritz et al. 2018). Other than in some systems with obvious visual sexual dimorphism, behavioral isolation is less thoroughly studied in lizards and snakes compared with many other animal taxa; hence, our understanding of speciation in this species-rich group is incomplete. Considering that these cryptic species complexes are by definition extremely similar in morphology, visual cues are unlikely to serve as mating signals. Chemical communication (i.e., pheromones), however, is important in the social interactions of many lizards and snakes (Mason and Parker 2010; Martín and López 2014). Pheromones influence intrasexual aggression (e.g., Martín et al. 2007; Khannoon et al. 2011), kin identification (e.g., Bordogna et al. 2016), and mate choice (e.g., Cooper and Vitt 1986; Shine et al. 2002; Martín and López 2004, 2006; Scott et al. 2015). In several other animal groups, divergent pheromones influence premating reproductive isolation, particularly among insects such as moths and drosophilid flies but also in vertebrates such as rodents, fish, birds, and reptiles (reviewed in Smadja and Butlin 2009). This is unsurprising given that olfaction appears to be the most ubiquitous sensory system among animals (Ache and Young 2005).

We test the hypothesis that pheromones have diverged among a cryptic species complex of Australian geckos. The Bynoe's gecko (*Heteronotia binoei*; Gray 1845) is a small, terrestrial, nocturnal lizard distributed widely across Australia (Wilson and Swan 2017). Although currently classified as a single species, *H. binoei* has long been recognized as a complex of several morphologically similar species (Moritz et al. 1990; Fujita et al. 2010). More recent multilocus sequencing across the range of *H. binoei* reveals that it comprises a dozen or more morphologically conservative but genetically distinct candidate species (Moritz et al. 2016). Species boundaries remain uncertain because genetic divergence between lineages varies across a broad spectrum, and

morphological characters (such as scalation and color pattern) are extremely variable within populations. Several genetic lineages of *H. binoei* are known to occur in sympatry or at parapatric contact zones with little or no genetic introgression (Moritz et al. 2016; C. Moritz, unpublished data; fig. 1A). Accordingly, we expect that premating isolating barriers will be present between overlapping lineages. Similar to many other lizards (García-Roa et al. 2017), *Heteronotia* possess glands that produce a waxy secretion via epidermal pores anterior to the cloaca (fig. 1B, inset). In *Heteronotia*, these epidermal pores are present only in males. Several studies in other lizards provide evidence that female lizards prefer to associate with the secretions of conspecific versus heterospecific males, suggesting a role for epidermal pore secretions in mate choice and behavioral isolation (reviewed in Martín and López 2011).

Considering these points and that many *H. binoei* lineages are visually indistinguishable, we predict that pheromones influence behavioral isolation in these lizards. If so, we expect the chemical blends of epidermal pore secretions to have diverged between lineages. We also predict that pheromones will have diverged to a higher degree than morphology, which would inform us about the speciation process and suggest that pheromones might be a useful trait to integrate into species delimitation. To test these hypotheses, we characterized morphology and the lipophilic fraction of epidermal pore secretions from 10 lineages of *H. binoei* occurring in three regions of sympatry in northeastern Australia, where the deepest phylogenetic structure occurs (Moritz et al. 2016). We then compared both the degree of pairwise overlap and mean pairwise distances for morphology and pheromone traits between lineages to test whether pheromones are more divergent than morphology. We hence demonstrate how this methodology could unlock cryptic species diversity in many lizard and snake groups.

Material and Methods

Field Sampling

Ten genetically divergent mitochondrial DNA (mtDNA) lineages (candidate species) of *Heteronotia binoei* were sampled across northern Queensland (fig. 1A) in November 2015 and November 2016, coinciding with the reproductive season of these geckos (S. Zozaya, unpublished data). The distribution of lineages through this region was largely already known from a combination of geographically extensive screening of mtDNA and eight nuclear DNA (nDNA) introns (Fujita et al. 2010; Moritz et al. 2016). For this geographic region in particular, there is strong concordance in major lineages detected by mtDNA and nDNA introns, and divergence among the candidate species we sampled is deep (~10%–20% for mtDNA; Moritz et al. 2016; see “Results”).

Figure 1: A, Distributions and pheromone sampling sites for the 10 lineages of *Heteronotia binoei* sampled in this study. Colors and shapes correspond to the symbols appearing next to the respective lineage in the phylogeny. Filled symbols represent sites where the respective lineage was sampled for pheromones, whereas open symbols show the known distributions for each lineage based on mitochondrial DNA (mtDNA) sequencing. Symbols are slightly offset at sites of sympatry so as not to obscure each other. Dashed circles depict the three regions of contact referred to in the article. B, Gas chromatograph trace of pheromones from the epidermal pores (inset) of a single male *H. binoei*. Individual peaks represent single compounds or groups of similar compounds and taken together form a pheromone blend. Numbered peaks are those used to assess multivariate pheromone divergence among lineages. Note that in this sample, peaks 3, 14, 16, and 18 are small. Prominent peaks lacking numbers are those identified as possible contaminants. C, Relationships among major mtDNA (*nd2*) lineages of *H. binoei* inferred using RAxML, with numbers showing bootstrap support values (%) for major nodes. The lineages sampled in this study are labeled, and those not sampled are collapsed where possible.

Our sampling focuses on three regions of contact where several candidate species co-occur in a mosaic of widely sympatric or narrowly overlapping lineages (fig. 1A). We briefly introduce each region below:

Northwest Queensland Region (NWQld Contact). Here we sampled three candidate species: the rock specialist NWQ and the two habitat generalists SM6NE and Gulf-W (fig. 1A). Although somewhat separated by habitat, NWQ is often sympatric with both SM6NE and Gulf-W, sometimes being found within the same retreat sites (S. Zozaya, personal observations). The SM6NE and Gulf-W lineages appear to have narrowly overlapping parapatric distributions.

Southern Cape York Peninsula Region (CYP Contact). Here we sampled four candidate species: CYA6N, CYA6S, EIU, and Gulf-E (fig. 1A). CYA6N and CYA6S are parapatric habitat generalists. EIU is mostly rock dwelling and is sympatric with the habitat generalists CYA6S in the south and Gulf-E in the north of its distribution, although all three have been recorded within the same retreat sites near Forsyth, Queensland. CYA6S is sympatric with Gulf-E at several sites.

Townsville Region (Tsv Contact). The Townsville region is a smaller area (~50 km radius) with three candidate species: EA6, Paluma, and MI (fig. 1A). EA6 is a widespread habitat generalist, occurring across eastern and southern Australia,

whereas Paluma and MI are sister lineages restricted to the Paluma Range and Magnetic Island, respectively. Notably, MI appears to be the most strongly rock-dwelling lineage of *H. binoei*. EA6 overlaps with the Paluma lineage along the eastern foothills of the Paluma Range. MI is currently isolated on Magnetic Island but likely occurred in contact with EA6 during lower sea levels.

We sampled one to five sites for each lineage depending on the number of known localities for each (some lineages are known from a few or single localities; e.g., MI and Paluma). We sampled a minimum of five male geckos at each site and up to 10 males at sites where the geckos were abundant. Geckos were captured at night and processed as follows: Tweezers were used to gently apply pressure around the epidermal pores to exude the waxy secretions. The secretions were then collected with a clean stainless steel probe and deposited into an individual glass gas chromatography microvial. Tweezers and probes were cleaned with analytical-grade isopropanol and wiped with a new Kimwipe before and after sampling a gecko. Each vial was sealed with a cap lined with polytetrafluoroethylene (PTFE) and stored in a -20°C freezer until analyzed. A control was collected at each site by following the same protocols as described above but not collecting any sample from a gecko, allowing us to identify contaminants incurred during sampling. Finally, a small section of tail tip was collected for mtDNA sequencing.

Lineage Identification and Relationships

For each site in this study, at least three individuals were sequenced for *nd2* (NADH dehydrogenase subunit 2) to confirm the identification of the lineages present. If, however, the site was known to or found to have more than a single lineage, all individuals were sequenced. DNA was extracted using the salting-out method of Sunnucks and Hales (1996) and amplified using the polymerase chain reaction (PCR) primers tRNAI and tRNAA from Strasburg and Kearney (2005). We followed the PCR protocols of Fujita et al. (2010). The resulting sequences were cleaned and aligned in Geneious version 6.1.8 (Drummond et al. 2008). The alignment was visually checked and verified by translating the *nd2* coding region into amino acids. These sequences were then aligned with a subset of the *nd2* alignment from Moritz et al. (2016) that included 136 sequences representing all major lineages of *H. binoei*, as well as six sequences of *Heteronotia planiceps* to serve as an outgroup. We used this combined alignment (231 sequences; see “Results”) to infer a phylogenetic tree using maximum likelihood with RAxML version 8.2.11 (Stamatakis 2014). We applied the GTRCAT approximation of rate heterogeneity and performed a rapid bootstrap analysis with 100 bootstrap replicates for statistical support. Sequences have been deposited in GenBank (accession nos. MK521075–MK521169).

Most of the mtDNA lineages have been found to be distinct for nuclear genes in previous multilocus analyses with extensive sampling of individuals (Moritz et al. 2016) and so represent candidate species. For three newly discovered mtDNA lineages from northeast Queensland (EIU, MI, and Paluma), our current sampling of individuals for nDNA is limited to one or two individuals per lineage, but this does confirm their phylogenetic distinctiveness (C. Moritz, unpublished data). Given their substantial mtDNA divergence and this preliminary nDNA data, we include these as candidate species here.

Morphological Data

For morphological analyses, we used measurements from 136 genetically identified (*nd2* sequenced) preserved specimens of the 10 lineages of *H. binoei* held at Australian National University and James Cook University (table S1; tables S1–S8 are available online). Specimens were not the same individuals sampled for pheromones but were collected from the same regions. All available adult specimens for each lineage were measured to the nearest 0.01 mm for eight linear morphological traits using Mitutoyo digital calipers. The traits were chosen as they have been shown to correlate with how lizards use their environment (Losos 2009). The traits were snout-to-vent length (SVL), interlimb length, head length, head width, snout length, eye diameter, hindlimb length, and forelimb length. See table S1 for details of how traits were measured and raw data.

Pheromone Characterization

Epidermal pore and control samples were characterized by gas chromatography in a randomized order. Each sample vial had 50 μL of *n*-hexane and 50 μL of N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) added. Hexane serves as the solvent, whereas BSTFA derivatizes the sample, allowing polar compounds to mix with the solvent. New caps with PTFE wafers were then used to seal each vial. Vials were then mixed on a vortex for 30 s and placed on a 70°C heat block for 60 min to promote derivatization. Vials were then mixed with a vortex for an additional 30 s following removal from the heat block. Samples were then immediately characterized on an Agilent 6890N gas chromatograph using an Agilent HP-5 30-m column with a diameter of 0.25 mm. An Agilent 7683 autosampler was used to inject 1 μL of sample into a 200°C pulsed-pressure splitless inlet. Due to a software error, only 0.5 μL was injected for those samples collected in November 2016 (see table S2); however, since we analyze pheromone composition as proportional data, this does not affect our analyses. The oven temperature program started at 50°C , then was ramped at 25°C per minute to 125°C , then was ramped at 15°C per minute to 325°C , and then was held for 8 min, for a total run time of 24.5 min. The flame ionization

detector was set at 250°C. Before and after sample injection, the autosampler syringe was cleaned with three washes of analytical-grade isopropanol followed by three washes of analytical-grade *n*-hexane. Chromatograms were manually integrated using Agilent OpenLab software. Chromatogram peaks that appeared in controls were considered to be contaminants and were excluded from integration of all samples. Pheromone data have been deposited in the Dryad Digital Repository (<https://dx.doi.org/10.5061/dryad.4h9170k>; Zozaya 2019).

Phenotypic Trait Analysis

We compared divergence of morphology and pheromone composition among the 10 lineages of *H. binoei* sampled. Our approach was to first compare these traits across all lineages and then repeat the analyses on subdivided data sets representing the three regions of contact (fig. 1A) to better detect fine-scale patterns of divergence among co-occurring lineages. Pairwise contrasts were carried out between lineages within each contact zone. We did not employ phylogenetic comparative methods here for two reasons. First, phylogenetic comparisons of trait evolution with only 10 taxa are not statistically sound (Blomberg et al. 2003). Second, such analysis is not important for answering our core questions: Are pheromone blends lineage specific, and are pheromone blends more divergent than morphology between candidate species? We recognize that phylogenetic comparative analyses are important for understanding the evolution of these traits, and work is underway incorporating data for more than 30 lineages across the *Heteronotia* radiation to investigate phenotypic trait evolution (S. Zozaya, unpublished data).

Morphological Traits. We performed a principal component analysis (PCA) on the eight morphological traits and retained the minimum number of PCs needed to account for approximately 100% of body size variation (PC1) and approximately 85% of the remaining body shape variation (table S3). All PCAs were performed using the *rda* function in the R package *vegan* using a correlation matrix (Oksanen et al. 2017). This was done for the complete data set of all 10 lineages and then repeated for individual data sets representing the three contact zones. Sex was initially included as a main effect and an interaction with lineage in the analysis of body shape outlined below, but both effects were not significant. Thus, we excluded sex and included both males and females in analyses of body shape to increase sample sizes.

Pheromone Traits. We successfully characterized pheromone samples from a total of 128 individual geckos representing the 10 genetically divergent lineages (table S2). A total of 25 peaks were integrated for all pheromone samples (fig. 1B; table S4). Each peak represents either a single com-

pound or two or more compounds with similar retention times. When similar compounds could not be reliably separated, they were considered as a single trait. Proportions were calculated using the total of all 25 integrated peaks as the divisor. We log-contrast transformed each peak to account for the unit-sum constraint of proportional data using $\log_{10}(\text{proportion peak } n / \text{proportion denominator peak})$ (Aitchison 1986). Peak 8 was selected as the denominator of the log-contrast transformation because it varies the least among lineages, resulting in 24 log-contrast pheromone traits. We corrected for variation in chemical characterization of pheromone composition between years by taking the residuals of a linear regression of each log-contrast trait against year (2015 or 2016). We used PCA (methodology as described above) to reduce the 24 log-contrast-transformed traits for multivariate analysis, keeping the minimum number of PC axes to account for approximately 85% of trait variation (table S5). This was done for the complete data set of all lineages as well as for each data set representing the three contact zones. For each contact zone comparison, however, we did not correct for year-to-year variation and instead used data collected only within the same field season (2015 for CYP and Tsv contacts, 2016 for NWQld contact). To ensure that our results were not an artifact of the log-contrast transformation method, we also reran all analyses with logit-transformed pheromone data (Warton and Hui 2011), which did not change our results (see table S6 for details and results of these analyses).

Testing for Phenotypic Trait Divergence among Lineages

To test whether phenotypes differed significantly among lineages, the following dependent variables were included in the multivariate analyses: for the overall comparison of all 10 lineages, PC1–5 representing 98% of morphological variation (~100% body size variation and 85% body shape variation) and PC1–9 representing 85% of pheromone variation were retained; for the NWQld contact, PC1–5 representing 97% of morphological variation and PC1–6 representing 82% of pheromone variation were retained; for the CYP contact, PC1–5 representing 96% of morphological variation and PC1–6 representing 84% of pheromone variation were retained; and for the Tsv contact, PC1–4 representing 98% of morphological variation and PC1–5 representing 85% of pheromone variation were retained.

To test the fixed effect of genetic lineage on morphology and pheromone traits, we carried out multivariate analyses using the MIXED procedure in SAS version 9.2. We carried out the formal significance testing using the MIXED procedure due to our unbalanced sampling design and to correctly specify the variance-covariance structure among variables. To obtain a multivariate significance test in the MIXED procedure, a likelihood ratio test is used to compare two models whose parameters are estimated using maximum likelihood:

the full model containing lineage (the lineage-by-trait model) versus the null model without lineage (the trait-only model; full details are in Wright 1998; e.g., Hoskin et al. 2011). We selected the variance-covariance structure for each analysis by running every full (lineage-by-trait) model with each of eight covariance structures available in the MIXED procedure (Kincaid 2007). We then compared the corrected Akaike information criterion (AICc) scores of the full models and selected the model that yielded the lowest AICc score (table S7; Fernandez 2007). Both full and null models were run using denominator degrees of freedom estimated using the Kenward-Rogers method due to unequal sample sizes among lineages (SAS Institute 2010). To obtain the likelihood ratio test (LRT), the difference in the $-2 \log$ likelihoods between the full and null models was tested against a χ^2 distribution, with degrees of freedom being the difference in the number of parameters between the two models (Wright 1998). If the effect of lineage is found to be significant using the LRT, then a model with planned contrasts comparing between lineages can be assessed. In MIXED, significance testing for planned multivariate contrasts is done using the full model and with parameters calculated using restricted maximum likelihood rather than maximum likelihood (Wright 1998). This then provides multivariate F -tests for pairwise comparisons between lineages for both morphology and pheromones within each contact region. All P values were globally adjusted for multiple comparisons using the false discovery rate method (Benjamini and Hochberg 1995).

To visualize the above formal statistical analyses, we calculated the first two canonical variates (CVs) from a multivariate linear model, which represent the linear combination of traits that differ most among the respective lineages (fig. 2, table S8). The models included the same parameters as each respective multivariate analysis described above but used the GLM procedure rather than the MIXED procedure in SAS, which cannot produce CVs. The first two CVs of the overall morphology analysis accounted for 57.17% (CV1) and 22.46% (CV2) of variation included in the model (total, 79.63%), and the first two CVs of the overall pheromone analysis accounted for 46.25% (CV1) and 24.42% (CV2) of variation included in the model (total, 70.63%). The percentages of variation included in the contact zone analyses are presented in table S8. To interpret which morphology and pheromone traits contributed to the divergence among lineages, we used the rule of thumb where traits contribute significantly to a CV if they have a loading of at least 70% of the absolute highest loading variable (Mardia et al. 1979; e.g., Blows and Higgie 2003; Hoskin et al. 2011). This identified which PCs contributed significantly to each CV (table S8). We then used the same approach to identify which traits contributed significantly to each relevant PC (morphology: table S3; pheromones: table S5).

Comparison of Trait Divergence

To test whether lineages differ relatively more in morphology or pheromones, we compared both pairwise trait overlap and mean pairwise trait distances for the two trait types. To compare trait overlap, we carried out three steps. First, we calculated the minimum convex hull polygons of these two CVs for each lineage from the overall analysis that included all 10 lineages. Second, the overlap area and union area (the area of the two respective polygons combined) were then calculated for each pairwise lineage comparison using the `gArea` and `gUnion` functions in the R package `rgeos` (Bivand et al. 2014). Finally, the proportional area of overlap for each lineage comparison was calculated by dividing the overlap area by the union area. Because of a large number of zero values (due to no polygon overlap between many lineages), we tested whether the degree of overlap among all 10 lineages differed significantly between traits via a nonparametric Kruskal-Wallis one-way ANOVA by ranks, with proportion of overlap as the response variable and trait type (morphology vs. pheromones) as the explanatory variable.

To compare trait distances, we took the average pairwise Euclidean distance between each lineage pair for both morphology and pheromones. For morphology, each variable was corrected for body size by taking the residuals of a linear regression of each trait against SVL, yielding seven size-independent measures of body shape. The eight morphological traits (SVL and seven size-corrected shape variables) were then standardized to a mean of 0 and a standard deviation of 1 to scale the two trait types. The 24 log-contrast pheromone variables were also standardized before analysis. For both morphology and pheromones, we then used the R package `vegan` to first calculate a Euclidean distance matrix between all observations with the function `vegdist`, followed by calculating mean pairwise distances between lineages with the function `meandist` (Oksanen et al. 2017). We used a t -test to test whether mean pairwise distances among the 10 lineages differed significantly for the two trait types (morphology vs. pheromones). To be conservative, we also reran this t -test comparison but with mean pairwise distances calculated with PC1–6 for each morphology (99% variation) and pheromones (76% variation) so that the same number of dimensions represented each trait type. This was in case the far higher number of dimensions for pheromones biased the result.

Results

Lineage Identification and Relationships

We successfully obtained approximately 919-bp sequences of *nd2* from 95 individuals of *Heteronotia binoei* sampled in this study. These, combined with the approximately 1,041-bp sequences from 136 individuals taken from Moritz

Figure 2: Morphology and pheromone blends show variation within and between the 10 candidate species of *Heteronotia binoei* examined here. Colors and symbols match those in figure 1. The two rows are trait space representations of the first two canonical variate axes (CV1 and CV2) of morphology (*top*) and pheromone composition (*bottom*). The first column shows trait values for all 10 lineages; symbols are centroids representing the mean of CV1 and CV2 for each lineage, with whiskers representing 95% confidence intervals. Subsequent columns represent the three regions of contact, with corresponding labels appearing above each column; symbols represent the trait values for individuals, and polygons represent minimum convex hulls for each lineage.

et al. (2016), yielded an alignment of 231 sequences. Phylogenetic analysis of these sequences confirms the lineage membership of geckos from which pheromones were sampled. All 10 candidate species sampled herein are deeply divergent mtDNA lineages with maximum likelihood bootstrap support values of 100% (fig. 1C). The major lineages recovered in this phylogeny are consistent with those of previous studies (Fujita et al. 2010; Moritz et al. 2016), although deep relationships vary. Additionally, our phylogeny includes three newly discovered divergent lineages from north-

east Queensland that are not in previous studies: EIU, MI, and Paluma.

Phenotypic Divergence among Lineages

Overall Lineage Comparison. Morphology and pheromone composition differed significantly among the 10 lineages of *H. binoei* (table 1). Figure 2 is a visual representation of phenotypic divergence among lineages, with the first column of panels representing the overall lineage comparison

Table 1: Results of multivariate analysis of morphological and pheromone divergence among 10 candidate species of *Heteronotia binoei* in northeastern Australia

Site (region of contact), trait, lineage	MANOVA			
	χ^2	<i>F</i>	df	<i>P</i>
All lineages:				
Morphology	234	...	45	<.001
Pheromone composition	450	...	81	<.001
NWQld contact (Gulf-W, NWQ, SM6NE):				
Morphology	40	...	10	<.001
Gulf-W vs. NWQ	...	4.53	5, 175	<.001
Gulf-W vs. SM6NE	...	2.03	5, 175	.076
NWQ vs. SM6NE	...	5.45	5, 175	<.001
Pheromone composition	49	...	12	<.001
Gulf-W vs. NWQ	...	3.45	6, 192	.002
Gulf-W vs. SM6NE	...	3.39	6, 192	.003
NWQ vs. SM6NE	...	5.34	6, 192	<.001
CYP contact (CYA6N, CYA6S, EIU, Gulf-E):				
Morphology	46	...	15	<.001
CYA6N vs. CYA6S	...	.15	5, 295	.980
CYA6N vs. EIU	...	4.08	5, 295	.001
CYA6N vs. Gulf-E	...	4.14	5, 295	.001
CYA6S vs. EIU	...	4.14	5, 295	.001
CYA6S vs. Gulf-E	...	3.87	5, 295	.002
EIU vs. Gulf-E	...	3.62	5, 295	.003
Pheromone composition	132	...	18	<.001
CYA6N vs. CYA6S	...	1.46	6, 140	.196
CYA6N vs. EIU	...	12.90	6, 140	<.001
CYA6N vs. Gulf-E	...	16.04	6, 140	<.001
CYA6S vs. EIU	...	6.65	6, 140	<.001
CYA6S vs. Gulf-E	...	10.51	6, 140	<.001
EIU vs. Gulf-E	...	16.18	6, 140	<.001
Tsv contact (EA6, MI, Paluma):				
Morphology	35	...	8	<.001
EA6 vs. MI	...	6.98	4, 128	<.001
EA6 vs. Paluma	...	2.06	4, 128	.090
MI vs. Paluma	...	3.73	4, 128	.006
Pheromone composition	32	...	10	<.001
EA6 vs. MI	...	2.61	5, 85	.030
EA6 vs. Paluma	...	4.13	5, 85	.002
MI vs. Paluma	...	2.70	5, 85	.026

Note: Results show the overall analysis of all lineages, as well as analysis for each of three regions of contact with pairwise comparisons. A likelihood ratio test is used to test for overall multivariate divergence among lineages. The likelihood ratio χ^2 value is the difference between the $-2 \log$ likelihoods of the full (lineage-by-trait) model and the null (trait-only) model. For the pairwise planned contrasts, significance testing is done within the full model using *F*-tests. The statistical significance for all *P* values was unchanged by false discovery rate adjustment. The unadjusted *P* values are reported here.

and subsequent columns representing each region of contact. Inspection of PCA and CV loadings (tables S3, S8) reveals that morphological divergence in the overall comparison is largely driven by body size (CV1) and eye diameter (CV2). For pheromone data, CV axes are influenced by many peaks (12 of 25), the majority of which have early retention times (tables S5, S8).

NWQld Contact. Both morphology and pheromone composition differed significantly among lineages at this contact zone, with overall body size and eye diameter being the main contributors to the divergence (tables S3, S8). Pairwise contrasts offer more detail regarding patterns of divergence (table 1; fig. 2): the rock-dwelling NWQ is smaller and has relatively larger eyes in comparison to both Gulf-W and SM6NE, while these two lineages are not significantly morphologically divergent from each other. Pheromone composition, however, differed significantly for all three pairwise contrasts, with eight peaks predominantly contributing to the divergence (tables S5, S8). All eight peaks had early or moderate retention times.

CYP Contact. Morphology and pheromone composition differed significantly among lineages (table 1; fig. 2). Morphology was significantly divergent between all lineages except for the comparison between CYA6N and CYA6S, with overall body size being the main contributor to the divergence (tables S3, S8). Similarly, pheromone composition differed significantly between all pairwise comparisons except between CYA6N and CYA6S. Ten peaks contributed to the divergence among lineages, with eight of those having early retention times (tables S5, S8). Notably, the closely related and parapatric CYA6N and CYA6S lineages do not differ in any of the phenotypic traits measured.

Tsv Contact. Morphology and pheromone composition differed significantly among lineages at this contact zone (table 1; fig. 2), with overall body size contributing most to the divergence (tables S3, S8). The large-bodied MI lineage is morphologically divergent from both EA6 and Paluma, although EA6 and Paluma are not significantly divergent from each other (table S3 and S8). Pheromone composition, however, differs strongly and significantly for all pairwise contrasts. Interestingly, only five compounds contribute strongly to divergence at this contact zone (tables S5, S8).

In summary, the pattern of morphological divergence is mostly consistent across analyses, with size being important in CV1 in the overall comparison and at all contacts and eye diameter being important in CV2 in the overall comparison and contributing at two of the three contacts (NWQld and Tsv but not CYP). The only common pattern in pheromone divergence is that many peaks are divergent among lineages (overall comparison: 12 peaks; NWQld: 8 peaks;

CYP: 10 peaks), with earlier retention times being generally more involved in divergence than those with later retention times. The exception is the Tsv contact, where only five peaks appear to be involved in the divergence among lineages and with no pattern of early or late retention times.

Comparison of Morphological versus Pheromone Divergence

Pheromone composition overlaps significantly less than morphology among the 10 lineages (Kruskal-Wallis test, $\chi^2 = 18.75$, $df = 1$, $P < .001$; fig. 3A). Similarly, mean pairwise distances among the 10 lineages are significantly greater than morphological distances, both when all variation is included (t -test, $t = -13.37$, $df = 83$, $P < .001$) and when the more conservative approach with PC axes is performed (t -test, $t = -7.34$, $df = 80$, $P < .001$; fig. 3B).

Discussion

Comparing phenotypic divergence among closely related species can reveal the traits influencing coexistence and reproductive isolation. While pheromones are known to be important in social interactions in many squamates, they have rarely been analyzed in terms of divergence between cryptic vertebrate lineages. This is because the collection of secretions and the analysis and interpretation of chemical signals they contain is difficult in comparison to morphology or other signaling modalities, such as vision or acoustics. A particular issue is the potentially enormous dimensionality of chemical signals, where a signal can range from a single compound to hundreds of compounds. Here we provide a methodology for field collection and subsequent analysis of complex pheromone blends and a framework for comparing divergence in pheromones and other traits. We addressed the issue of dimensionality by performing data reduction via PCA and by using a conservative approach that biases comparisons of trait divergence in favor of morphology over pheromones. Furthermore, we analyzed divergence across multiple regions of contact to assess consistency in our results.

We found substantial pheromone divergence between genetically divergent lineages of *Heteronotia binoei*. On average, overlap in pheromone composition between lineages is less than morphological overlap, and mean pairwise phenotypic distances between lineages are greater for pheromones than morphology (figs. 2, 3). Furthermore, while morphological divergence is idiosyncratic, pheromone blends are consistently divergent among all pairwise comparisons except between CYA6N and CYA6S. Importantly, at all three contact regions we found very little overlap in pheromones between lineages. The observation of lineage-specific pheromone blends, particularly in areas of overlap, and that pheromones have diverged more than morphology meet

Figure 3: Pheromone blends have diverged relatively more than morphology among the 10 candidate species of *Heteronotia binoei* examined here, with less overlap in trait space (A) and greater distance between lineage means (B). *A*, Overlap analysis. Boxplots illustrate the proportion of pairwise trait overlap among lineages of *H. binoei*. The more different these traits are between lineages, the lower the value of overlap. *B*, Distance analysis. Boxplots illustrate the mean pairwise Euclidean trait distance among lineages of *H. binoei*. The more different these traits are between lineages, the higher the distance value. Boxplots show medians as horizontal black lines, interquartile ranges (IQRs) around the medians as boxes, nonoutlier ranges (within 1.5 times the IQR) as whiskers, and outliers (>1.5 times the IQR) as individual data points.

our prediction that pheromones influence behavioral isolation in this cryptic species complex.

Although less divergent than pheromones, there is significant morphological divergence among lineages. Morphological differences are known to reflect ecological divergence in lizards, such as structural habitat use and prey type (Losos 2009; Moritz et al. 2018). Such divergence can reduce competition and facilitate coexistence (Schluter 2000) and may directly play a role in mate choice and reproductive isolation (e.g., Richmond and Jockusch 2007). Morphological divergence in the *Heteronotia* lineages examined here is difficult to interpret. First, morphology is sometimes divergent but with considerable overlap between lineages. Pairwise contrasts show that morphological divergence is most often driven by body size. We see no clear interpretation of body size divergence (e.g., rock-dwelling lineages are both large and small), but our study was not designed to test this in detail. The other morphological trait contributing to lineage divergence in some analyses was larger eyes associated with rock-dwelling lineages (NWQ, EIU, and MI). This is interesting because a tendency toward larger eyes has been observed in other rock-dwelling geckos from northeastern Australia (e.g., Hoskin and Couper 2013). More research is necessary to assess whether morphological variation reflects ecological divergence in *H. binoei*.

The importance of pheromone divergence and behavioral isolation has long been recognized among invertebrate biologists (Smadja and Butlin 2009). For example, the phero-

mones of some closely related moths share the same major chemical components but differ in the blend of these components, contributing to the maintenance of reproductive isolation (Linn and Roelofs 1995). Similarly, differences in the blend of cuticular hydrocarbons influence premating isolation in various drosophilid flies (e.g., Blows and Allan 1998; Higgie et al. 2000). It is now evident that pheromones influence reproductive isolation in many vertebrate groups as well (Smadja and Butlin 2009), including lizards. Behavioral studies suggest that several lizard and snake groups use pheromones when choosing mates and that signals and preferences can vary among populations within and between species (Barbosa et al. 2006; Gabriot et al. 2012; García-Roa et al. 2016). Even though relatively few species have been studied, these taxa—snakes (Mason et al. 1989; Shine et al. 2002), skinks (Cooper and Vitt 1986, 1987; Scott et al. 2015), iguanians (Escobar et al. 2003; Labra 2011), and lacertids (Martín and López 2004)—are distributed across the clade of squamate reptiles, suggesting that pheromones are a common signaling trait across this group.

Of particular interest are lizard groups that possess epidermal pores. These are absent in snakes and skinks but present in lizard groups such as lacertids, tropidurids, and gekkonids (the gecko family containing *Heteronotia*). Behavioral studies of lacertid and tropidurid lizards suggest that the lipophilic fraction of epidermal pore secretions function both in a mate choice context and in male-male interactions (reviewed in Martín and López 2014). Epidermal pore

secretions contain dozens of compounds, which makes teasing apart the influence of individual compounds difficult and potentially irrelevant, given that it is likely that combinations of compounds are perceived in animals (Firestein 2001). Behavioral assays suggest that cholesta-5,7-dien-3-ol, ergosterol, and oleic acid influence female choice in lacertid lizards (Martín and López 2006, 2010) and that cholesterol, hexadecanol, and other long-chain alcohols influence male-male interactions (Martín and López 2007, 2008; Khannoon et al. 2011). Several of these or similar compounds are present in the epidermal pore secretions of most lizard species characterized to date (Weldon et al. 2008; Martín and López 2014), including geckos (Khannoon 2012). Preliminary analysis confirms that some of these compounds—such as cholesterol (peak 11, fig. 1B)—are present in the secretions of *H. binoei*; however, full characterization has been hampered because *H. binoei* produce very small volumes of secretion (S. Zozaya, unpublished data). Nevertheless, our results show that candidate species within *H. binoei* differ in the relative proportion of shared compounds. Considering this and that epidermal pore secretions influence mate choice and intrasexual competition in other lizards, we predict that behavioral experiments will reveal similar roles in *Heteronotia*. Our results here suggest that it is a combination of many pheromone compounds—including both minor and major compounds—that differ among lineages; therefore, it is likely that a blend of compounds mediates behavioral isolation among these cryptic lineages.

Interpreting the significance of pheromone divergence is difficult without an understanding of how different compounds influence behavior and the selection pressures acting on the evolution of pheromone blends. Given the hugely multidimensional nature of chemical signals, determining the specific influences of individual compounds on behavior might often be impossible; nevertheless, identifying the processes influencing pheromone evolution is possible (Yohe and Brand 2018). Pheromone divergence among populations and species can result from several processes, including divergent natural selection to different local environments (Endler 1992; Boughman 2002); selection to reduce maladaptive hybridization between sympatric species (i.e., reinforcement; Howard 1993; Higgie et al. 2000; Hoskin et al. 2005; Dyer et al. 2014), as well as other types of reproductive interference (Hoskin and Higgie 2010); divergent sexual selection, possibly due to female preference, male-male competition, or both (Andersson 1994; Nosil et al. 2007); and stochastic processes, such as drift and founder effects (Lande 1981). Divergent natural selection under local environmental conditions can play a role in the evolution and divergence of pheromones (Yohe and Brand 2018). Pheromones are a chemical signal, and a signal must be transmitted effectively in its environment if it is to be reliably detected by the intended receiver (Endler 1992). Thus, environmental conditions can exert selection pressure to optimize signal efficacy against

background conditions (e.g., sensory drive; Boughman 2002). For example, Martín et al. (2015) provide evidence that differences in pheromone blends between two populations of the Iberian wall lizard (*Podarcis hispanicus*) reflect adaptation to local environments. A substantial influence of climate on the evolution of epidermal pore secretions is further supported by a comparative analysis of 64 species of lacertid lizards (Baeckens et al. 2017), which showed that the pheromone secretions of species from more arid areas have higher proportions of stable fatty acid esters and high molecular weight alcohols. While our study does not address the influence of environment on pheromone composition, our results show that pheromones have diverged even among sympatric lineages of *H. binoei*, which suggests that abiotic factors alone cannot account for the degree of pheromone divergence observed here. Detailed behavioral data and phylogenetic comparative analyses will be key to interpreting the full significance of pheromone variation and divergence among populations and candidate species within this complex.

Understanding Cryptic Species Diversity

Cryptic species pose particular challenges to taxonomists and therefore to biodiversity and extinction estimates. Knowledge of sexually selected signals has been crucial for understanding species boundaries and diversification in many morphologically conservative groups. Taxonomic analyses of orthopteran insects (e.g., Nityananda and Balakrishnan 2006), frogs (e.g., Padial et al. 2008), mormyrid fish (e.g., Crawford and Hopkins 1989), and many other groups have relied heavily on the analysis of signaling traits. Analyses of these traits have provided information about reproductive isolation and the processes that drive it and have facilitated the description of many otherwise indistinguishable species. Without knowledge of these signals, the taxonomy of many groups would be poorly resolved.

Our results contribute to the understanding of signaling traits in squamate reptiles, the most species-rich group of terrestrial vertebrates. Studies of lizard signals have focused heavily on visual ornaments and displays, such as sexually dimorphic coloration and stereotypical movements (Pough et al. 2015). These studies have been important for understanding diversification and behavior in many lizard groups (e.g., *Anolis*; Losos 2009). We suspect that the study of pheromones will contribute similarly to our understanding of lizard groups lacking such visual displays, such as the *H. binoei* complex examined herein. Taxonomic resolution of the *H. binoei* complex is difficult because morphology in this radiation is mostly uninformative, coloration is variable and overlapping among most lineages, and genetic divergences vary across a broad spectrum (fig. 1C; Fujita et al. 2010; Moritz et al. 2016). Genetic analyses can be valuable for

estimating whether sympatric or parapatric populations are reproductively isolated but are more ambiguous for those that are allopatric (Singhal et al. 2018). For *H. binoei*, nearly all lineages examined to date with multilocus sequence data are statistically supported as separately evolving lineages (Fujita et al. 2010; Moritz et al. 2016; C. Moritz, unpublished data). Our chemical analysis indicates that pheromones are typically divergent among candidate species, even some that are relatively closely related (e.g., EA6, MI, Paluma). With more research and methodological development (outlined below), pheromone data can be integrated into a taxonomic framework to better infer species boundaries, particularly when morphology is highly conservative (Padial et al. 2010). Explicitly testing for reproductive isolation is arduous and often impractical, but analysis of mating traits—particularly those influencing reproductive isolation—can be a more practical alternative to infer species boundaries.

Future Directions

Pheromone divergence needs to be further linked to reproductive isolation in this system, and there are several ways to test this. First, behavioral studies can test the expectation that pheromones will be involved in mate choice and/or male competition. Second, the degree of pheromone divergence and contemporary gene flow can be assessed across the many pairwise contacts between lineages, with the expectation that greater pheromone divergence will correlate with lesser gene flow even after controlling for genetic distance. Third, assessing relative pheromone divergence in sympatric versus allopatric populations would test for an expected pattern of reproductive character displacement where lineages overlap. Finally, phylogenetic comparative methods can be used to test the expectation of more rapid divergence of pheromones compared with other traits, such as morphology, across the more than 30 lineages of the *Heteronotia* radiation across Australia.

The other avenue for further research in this system is investigating the genetic and environmental contributions to pheromone variation. As outlined above, our discovery of pheromone differences between lineages that are in sympatry and sampled at the same time suggests a strong genetic component. However, pheromone blends in lizards can be influenced by factors such as diet and thermal environment (Heathcote et al. 2014; Martín and López 2014). Resolving the role of plasticity due to the environment could involve correlating within-lineage variation in pheromone blends with abiotic variables, phylogenetic comparative analyses including abiotic variables, and estimating pheromone heritability using laboratory-reared families.

Beyond this system, studies are needed across more taxa to understand the importance of pheromones in population divergence and speciation (Symonds and Elgar 2008;

Smadja and Butlin 2009). Chemical signals are particularly complex to measure, analyze, and interpret, and hence pheromones have been studied relatively less than visual or acoustic signals in vertebrates (Symonds and Elgar 2008; Smadja and Butlin 2009). This in part explains why major groups of vertebrate diversity (e.g., many lizard clades) remain poorly resolved. Pheromones may also play a significant role in animal groups that rely primarily on visual or acoustic communication (e.g., frogs; Byrne and Keogh 2007), and future research may find an important role of pheromones in reproductive isolation in these groups. Although studying pheromones is difficult, they offer exciting opportunities to further resolve vertebrate diversity and the underlying processes generating it (Smadja and Butlin 2009).

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An adult male Bynoe's gecko (*Heteronotia binoei*) of the NWQ lineage. The pheromones of this deeply divergent genetic lineage have diverged substantially from other co-occurring lineages of Bynoe's gecko. Photo credit: Stephen M. Zozaya.